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Receptor-Mediated Endocytosis of Glucagon in Isolated Mouse Hepatocytes

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ABSTRACT The binding of glucagon to the cell surface and the pathway of intracellular transport of the hormone in isolated mouse hepatocytes were studied by autoradiography, colloidal gold-labeled glucagon (Au-glucagon), and biochemical methods. In cells incubated with ^{125}I-glucagon at 4°C, the label was mainly localized to the plasma membrane even after 60 min of incubation. At 20°C, the labeled ligand was internalized by the cells and the amount of internalized ligand increased with time of incubation. At 37°C, the ligand was rapidly internalized and found to be associated with coated or uncoated vesicles. Au-glucagon experiments revealed clearly the process of internalization of glucagon. Au-glucagon bound to the plasma membrane was transported to coated regions and then internalized into vesicles via coated pits. Biochemical results supported these findings from autoradiography and Au-glucagon experiments. Thus, glucagon is internalized by hepatocytes via receptor-mediated endocytosis.

Glucagon has been supposed to be internalized by cells via receptor-mediated endocytosis (Barazzone et al., 1980) like a number of other polypeptide hormones, growth factors, and macromolecules (Ashwell and Morell, 1974; Dickson et al., 1981; Kahn et al., 1981; Kaplan, 1981; Pastan and Willingham, 1981; Schlessinger et al., 1978; Willingham et al., 1981; Harding et al., 1983; Dautry-Varsat et al., 1983). Recently, Ackerman et al. (1983) showed evidence for receptor-mediated endocytosis of glucagon in leukocytes using colloidal gold-labeled glucagon. However, the process of binding of glucagon to the cell surface and the pathway of intracellular transport of the hormone in hepatocytes of the liver, the major target organ, has not been fully elucidated yet. There have been a few reports on autoradiographic analysis of the process in hepatocytes (Barazzone et al., 1980; Watanabe et al., 1982; Asada-Kubota et al., 1983). However, these authors have been unable quantitatively to associate labeled glucagon with structures showing morphologic characteristics of endocytic vesicles.

In the present study, therefore, we examined the process of binding and internalization of glucagon in isolated mouse hepatocytes not only by autoradiography but also colloidal gold-labeled glucagon and biochemical methods.

MATERIALS AND METHODS

Male ddY mice, 8–10 weeks old, were used. They had free access to food and water prior to the experiments. Cells were isolated from livers by perfusion of a collagenase containing solution via the portal vein (Klauning et al., 1981) under Nembutal anesthesia. The parenchymal cells were separated from nonparenchymal cells by four centrifugations at 50g for 1 min at 4°C (Watanabe et al., 1983). Isolated hepatocytes were suspended in Hanks' solution (pH 7.4) containing 1% bovine serum albumin (BSA) at 4°C prior to incubation and used within an hour after isolation.

Autoradiography

Isolated hepatocytes (5×10^5) were suspended with 0.4 nM ^{125}I-glucagon (New England Nuclear, Boston, MA; specific activity of 80–170 μCi/μg) in 1 ml of Krebs-Ringer bicarbonate buffer (pH 7.5) containing 3% BSA (Sigma) and 0.6 mM bacitracin and incubated at 4 or 37°C in plastic tubes. The

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incubation was performed in duplicate. Nonspecific binding was estimated by adding 50 μg of unlabeled glucagon to the medium. After incubation for 3, 10, or 30 min, 1 ml of cold Krebs-Ringer bicarbonate buffer (pH 7.5) was added to the incubation medium, the mixture was centrifuged at 100g for 30 sec at 4°C, and the supernate was quickly removed. The cell pellets were washed with the cold buffer by four suspensions and centrifugations at 100g for 1 min at 4°C. The cell pellets were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hr at 4°C, washed in 0.1 M phosphate buffer (pH 7.4) containing 5% sucrose for 30 min at 4°C, and postfixed in cold buffered 2% osmium tetroxide for 1 hr. The pellets were dehydrated with graded ethanol and embedded in Spurr's resin. Thin sections cut from the Spurr blocks were placed on collodion coated copper grids, stained with uranyl acetate, and coated with evaporated carbon, approximately 5 nm in thickness. The grids were coated with Sakura NR-H2 emulsion by a wire loop method (Caro et al., 1962), exposed for 6 weeks at 4°C, and developed with Konidol X. The sections were stained with lead citrate and examined on a Hitachi H-700H electron microscope.

Preparation of Colloidal Gold-Labeled Glucagon (Au-Glucagon)

Gold particles, 20 nm in average diameter, were made as described by Frens (1973). Glucagon-BSA conjugate was prepared according to the method of Ackerman et al., (1983). Adsorption isotherms were used to determine optimal concentrations for labeling. Glucagon-BSA-colloidal gold complex (Au-glucagon) was prepared from the glucagon-BSA conjugate and colloidal gold according to the methods of Ackerman et al. (1983). However, colloidal gold solution adjusted to pH 7.5 was used because the Au-glucagon showed stability between pH 7.0–8.0. Three milliliters of Au-glucagon thus prepared was suspended in 30 ml of 0.005 M sodium chloride containing 0.12% polyethylene glycol (MW 20,000), and centrifuged at 10,000g for 1 hr at 4°C to eliminate free glucagon, BSA, and glutaraldehyde. The precipitate, Au-glucagon, was suspended in 6 ml of 0.05 M Tris-HCl buffer (pH 7.6) containing 4% polyvinyl pyrrolidone, 3% BSA, 0.6 mM bacitracin, and 0.12% polyethylene glycol. Polyvinyl pyrrolidone, BSA, and polyethylene glycol were used for adjusting the osmolality of this solution, for blocking nonspecific bind of Au-glucagon to albumin receptors on the plasma membrane, and for stabilizing the gold particles, respectively. ^{125}I-glucagon-BSA-colloidal gold complex was also prepared to test the effectiveness of conjugation of glucagon and BSA. One milliliter of the Au-glucagon solution contained approximately 10 μg glucagon and about 110–150 molecules of glucagon were bound to one gold particle. The number of glucagon molecules bound to one gold particle was calculated as follows. The radioactivity of Au-glucagon was measured with an auto gamma counter (Packard, 5330). The radioactivity was divided by gram molecular weight of glucagon and multiplied by Avogadro's number to obtain the number of glucagon molecules per milliliter of the labeled colloidal gold. The number of gold particles per milliliter of the original unbound colloid was calculated by the method of Ackerman et al. (1983).

Binding and Internalization of Au-Glucagon

The cells (5×10^5) were suspended in 1 ml of the Au-glucagon solution and incubated for 3,5,10, or 30 min at 4°C, or 3,5, or 10 min cat 37°C. As control, the cells were incubated in the solution containing excess (300 μg/ml) unlabeled glucagon. Gold-labeled BSA (Au-BSA) was also used as a control. Au-BSA was prepared by exposing BSA. 0.05% glutaraldehyde prior to colloidal gold labeling and then by labeling according to the method of Geoghegan and Ackerman (1977). After incubation in Au-glucagon solution, the cells were washed twice with cold 0.1 M Tris-HC1 buffer (pH 7.6) containing 5% sucrose by centrifugations at 100g for 1 min at 4°C and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at 4°C. The cells were postfixed in cold buffered 1% OsO_4 for 1 hr, dehydrated, and embedded in Spurr's resin.

Biochemical assay of cell-associated Au-glucagon was performed using colloidal gold-labeled ^{125}I-glucagon (Au-^{125}I-glucagon). Cells (5×10^5) were iucubated in 1 ml of the medium containing 3 nM of Au-^{125}I-glucagon or gold-free ^{125}I-glucagon at 37°C, washed as described above, and cell-associated radioactivity was determined in an auto gamma counter.

Morphometry of Grains and Gold Particles

The grain distribution histograms were obtained according to the method of Barazzone et al. (1980). Six Spurr blocks were used per each time point examined. Six sections were cut from each block and two photographs of midplane sections of hepatocytes were taken from each section at × 5,000. Ten of the 12

photographs were used for the analysis, and the distance between grain center and the plasma membrane was measured.

To establish whether internalized grains were preferentially associated with lysosomes, the probability circle method (Stäubli et al., 1977) was used. About 130 grains were photographed at × 10,000 from six sections cut from three blocks. The half distance (280 nm) was derived from the distribution of ^{125}I-irradiation around a defined line source obtained from Na ^{125}I injected mouse thyroid gland according to the method of Bergeron et al. (1977). The total points accumulated in lysosomes or whole cytoplasm were counted and expressed as a percentage of the total number of grains counted. The volume density of lysosomes, calculated by the method of Weibel (1979), was used as reference for the grain counting.

Number of Au-glucagon bound to the cells was determined by the following method. Four blocks were randomly selected per each time point examined. One section was cut from each block, and ten photographs of a portion of the cells (one portion per cell) containing the plasma membrane were taken at × 10,000 from the section. The photographs were enlarged to a final magnification of × 20,000. Five of the ten photographs were chosen at random, and cytoplasmic area in each photograph corresponding to an area of 440 μm^2 was estimated using the point counting method (Weibel, 1979). The number of gold particles localized both along the plasma membrane and within the cytoplasm were counted. Thus, the number of gold particles per 100 μm^2 cytoplasm was calculated. Further, the number of gold particles along coated or uncoated regions of the plasma membrane and of the particles in vesicles or cytoplasmic matrix were counted. The values were epxressed as a percentage of total number of the particles.

Incubation and Assay of Cell-Associated Radioactivity

Isolated hepatocytes were incubated with 0.4nM ^{125}I-glucagon for 3,5,10,20,30,45,60, or 90 min at 4, 20, or 37°C, washed as described above, and then cell-associated radioactivity was determined in a gamma counter.

Assay of Cell Surface and Intracellular Radioactivity

The cells, incubated with 0.4nM ^{125}I-glucagon for 3,5,10,20,30,45, or 60 min at 4, 20, or 37°C, and washed as described above, were immersed in 1 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.05% trypsin and 0.9% sodium chloride for 30 min at 4°C. The cells were centrifuged at 500g for 10 min at 4°C, and radioactivity of the supernates and cell pellets was determined in a gamma counter.

Assay of Glucagon Degradation

The supernates, removed after incubation with ^{125}I-glucagon, were poured into 2 ml of cold 10% trichloroacetic acid (TCA), shaken for a few seconds using a Vortex mixer, centrifuged at 500g for 15 min at 4°C, and the radioactivity of TCA soluble and TCA precipitate fractions were determined in a gamma counter.

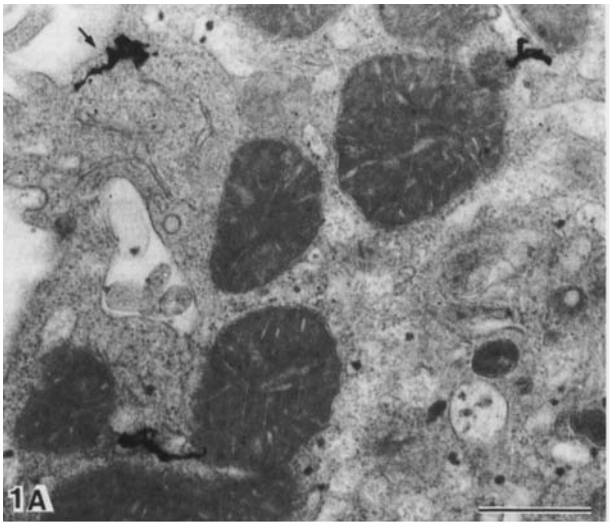
RESULTS

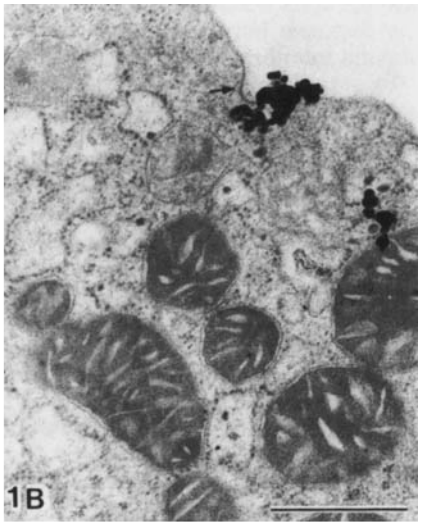
About 98% of the isolated cells obtained finally were parenchymal cells as determined by phase contrast microscopy. The average number of parenchymal cells yielded from a liver was 8×10^7. On the basis of trypan blue exclusion, about 90% of the cells were viable.

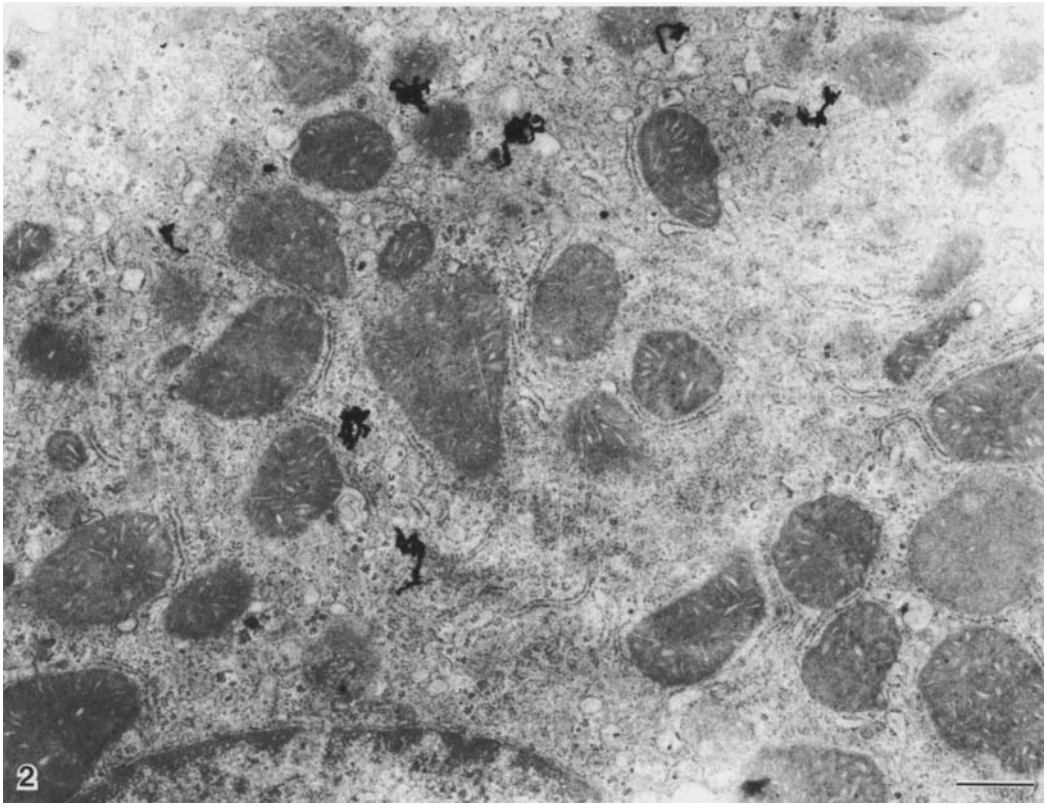
Autoradiography

At 4°C, developed grains were mainly localized to the plasma membrane, although the number of grains increased with time of incubation. Very few grains were observed over the cytoplasm and nuclei. In the cells incubated at 37°C for 3 min, although some grains were associated with the plasma membrane, most grains were already present over the cytoplasm. The internalized label was mainly localized to coated or uncoated vesicles and sometimes to other cell organelles and ctyoplasmic matrix (Fig. 1A,B). At 10 min of incubation, the distribution of the grains was similar to that at 3 min (Fig. 2). At 30 min, the grains were not exclusively localized to any specific organelles and were decreased in number (Fig. 3). During the incubation, very few grains were observed over nuclei. In the cells incubated with excess unlabeled glucagon, a few grains were occasionally seen along the plasma membrane and over the cytoplasm.

The grain-distribution histograms showed that at 4°C the grains were almost exclusively bound to the plasma membrane during 30 min of incubation (Fig. 4A). However, relatively much labeled material was already internalized by the cells incubated at 37°C for 3 min (Fig. 4B). The nonspecific binding and background was less than 15% of the total grains.







Figs. 1-3. Autoradiograms of portions of isolated mouse hepatocytes incubated with 0.4 nM ^{125}I-glucagon at 37°C.

Fig. 1A,B. Incubated for 3 min. The labels are already internalized into the cytoplasm. However, tangential sections through the plasma membrane infoldings could account for cytoplasmic label. Some of the grains are seen associated with coated regions of the plasma membrane (arrows). A, ×30,000. B, ×36,000. Bars = 0.5 μm.

Fig. 2. Incubated for 10 min. The grains are seen associated with coated or uncoated vesicles. ×18,500. Bar = 0.5 μm.

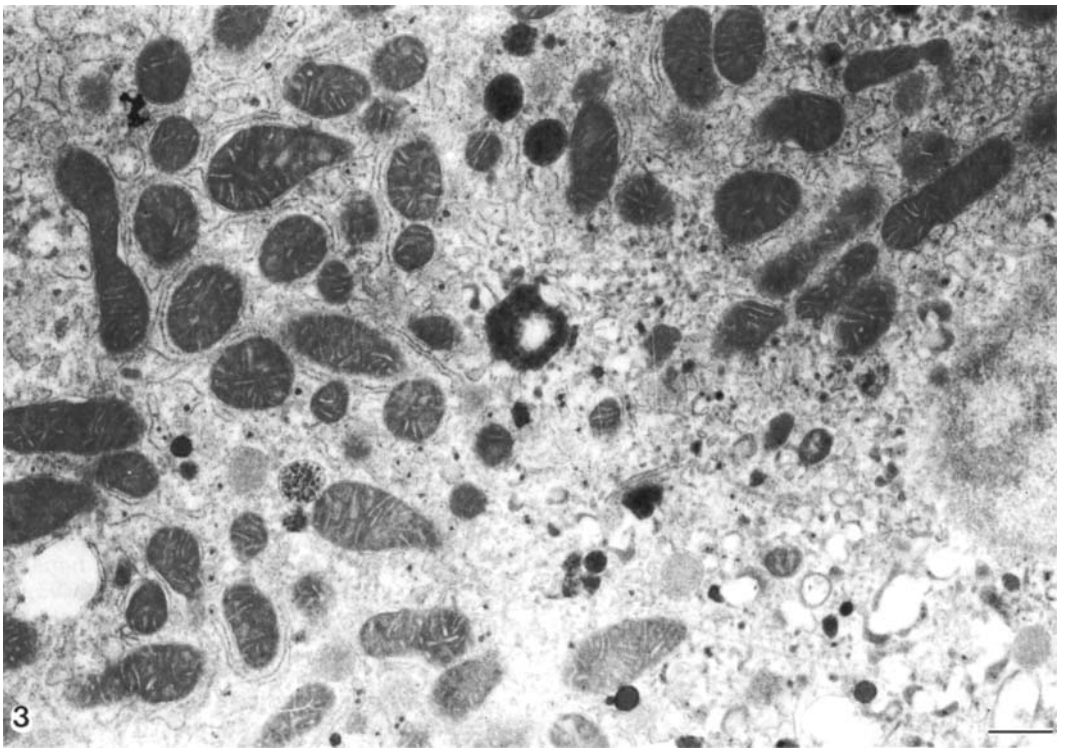


Fig. 3. Incubated for 30 min. The grains decrease markedly in number. Although many lysosomes are observed, the grains are not seen associated with them. ×15,000. Bar = 0.5μm.

The grains in the cells incubated at 37°C for 3, 10, or 30 min, analyzed by the probability circle method (Stäubli et al., 1977), were not exclusively localized to lysosomes (Table 1).

Au-Glucagon

By both visual inspection and quantitative analysis, Au-glucagon was localized on the plasma membrane during 30 min of incubation in the cells incubated at 4°C (Table 2). At 37°C, Au-glucagon was localized on the plasma membrane at 3 min of incubation. After 5 min, the ligand was internalized into the cytoplasm via coated pits (Fig. 5A,B), and about 15% of Au-glucagon was internalized and localized within the vesicles at 10 min (Fig. 5C).

To determine whether Au-glucagon was localized to coated regions of the plasma membrane, gold particle distribution was analyzed (Table 2). At 4°C, Au-glucagon was randomly bound to the plasma membrane. However, at 37°C, 20–35% of Au-glucagon was localized to coated pits and coated vesicles.

When the cells were incubated with Au-BSA, very low binding of gold particles to the plasma membrane was observed. In the cells incubated with Au-glucagon and excess unlabeled glucagon (300 μg/ml), very few gold particles were seen.

Binding of Au-^{125}I-glucagon to the cells was inhibited by excess unlabeled glucagon, indicating that the binding was specific (Fig. 6). The binding of Au-^{125}I-glucagon was lower than that of gold-free ^{125}I-glucagon.

Biochemical Results

Radioactivity associated with the cells.

When the cells were incubated at 4°C, the ligand was slowly bound, and the steady-state binding was reached at 60 min and maintained at least until 90 min (Fig. 7). At 20°C, high steady-state binding was reached at 30 min. At 37°C, the ligand was rapidly associated with the cells, showing a peak of binding between 3 and 20 min. About 7–15% of the total binding was nonspecific at each time point of incubation.

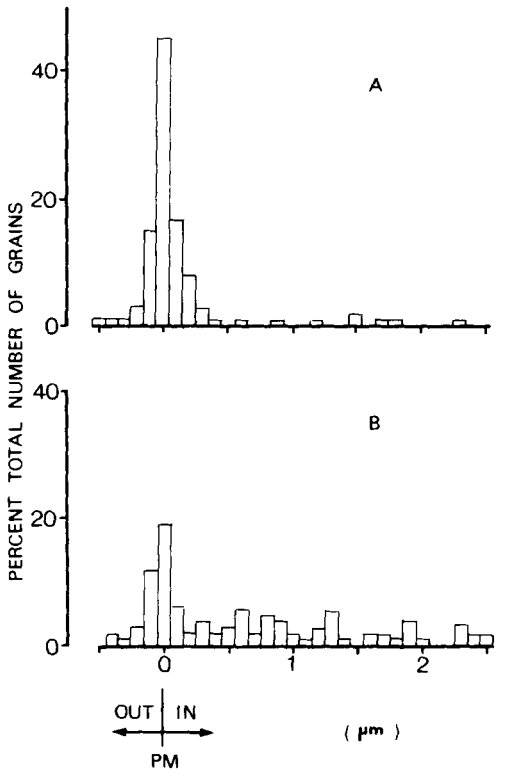


Fig. 4A,B. Grain distribution histograms of ^{125}I-glucagon binding to hepatocytes after incubation with 0.4 nM ^{125}I-glucagon at 4°C for 30 min (A) or at 37°C for 3 min (B). The grains within 2.5 μm from the plasma membrane were analyzed. The distance between the grain center and the plasma membrane was measured, and the percentage of total number of the grains was plotted as function of the distance. Histograms from the cells incubated at 37°C for 10 or 30 min are similar to this histogram (B). PM, plasma membrane.

Cell surface and intracellular radioactivity

At 4°C, all of the bound ligand was tryspin sensitive even after 60 min of incubation, indicating that all ligand was bound to the cell surface (Fig. 8A). At 20°C, the amount of trypsin-resistant ligand increased with time of incubation, and about 15% of the ligand was trypsin resistant at 60 min of incubation (Fig. 8B). At 37°C, about 20% of the ligand was found to be already trypsin resistant at 3 min; then the amount of internalized ligand did not change, although that of trypsin-sensitive ligand decreased (Fig. 8C). Thus, internalization of the ligand occurred at 20 and 37°C.

Glucagon degradation

At 4°C, ^{125}I-glucagon was not degraded (Table 3). At 20°C, the radioactivity appeared in a TCA solube fraction, and at 37°C about one half of the added ligand was degraded by the cells by 30 min.

DISCUSSIONS

As revealed in the present autoradiograms and grain-distribution histograms, labeled glucagon is localized to the plasma membrane of hepatocytes during incubation at 4°C, and most labels are internalized by the cells at 37°C. This result is consistent with observations of Barazzone et al. (1980) on isolated rat hepatocytes and of Watanabe et al. (1982) and Asada-Kubota et al. (1983) on hepatocytes of intact mouse liver. However, the internalized grains are associated preferentially with coated and uncoated vesicles in the present results. The apparent localization of the label to the vesicles in hepatocytes was not described in the previous reports (Barazzone et al., 1980; Watanabe et al., 1982; Asada-Kubota et al., 1983). It is suspected that glucagon bound to the plasma membrane is internalized into the vesicles.

The qualitative and quantitative results of Au-glucagon experiments reveal more accurately the process of binding of glucagon to hepatocyte surface and of the internalization of the ligand by the cells. Au-glcaugon bound to the plasma membrane is transported to the coated regions and then translocated into the cytoplasmic vesicles via coated pits. Therefore, glucagon is internalized by hepatocytes via receptor-mediated endocytosis. Thus, morphologic evidence of the internalization of glucagon by hepatocytes via receptor-mediated endocytosis was obtained for the first time by the use of the Au-glucagon method. Generally, it appears difficult to document the process of receptor-mediated endocytosis by autoradiography. Barazzone et al. (1980) reported that only less than 1% of the grains analyzed were associated with coated regions. However, the present results show that more than 18% of gold particles seen on the plasma membrane, was bound to coated regions.

The present biochemical results support the findings of the autoradiography and Au-glucagon experiments. Further, the results show that the ligand is internalized more slowly at 20°C than at 37°C, and, therefore, the internalization of glucagon by hepatocytes is temperature dependent.

TABLE 1. Relationship of the grains to lysosomes[1]

Time (min)	No. of grains analyzed	No. of test points on whole cytoplasm (A)	No. of test points on lysosomes (B)	$\frac{B}{A}=C$	Volume density of lysosomes (D)	C/D
3	132	629	8	0.0127	0.0151	0.84
10	127	611	7	0.0115	0.0124	0.93
30	129	635	14	0.0220	0.0289	0.76

[1]The grains in the cells incubated at 37°C for 3, 10, and 30 min were analyzed as described in the text. Volume density: μ^3/μ^3 cytoplasm.

TABLE 2. Distribution of Au-glucagon and Au-BSA in isolated mouse hepatocytes[1]

Ligands	No. of counted particles	No. of particles per cell sectioned (No./100 μm^2 cytoplasm)	Plasma membrane bound		Internalized	
			Uncoated (%)	Coated (%)	Vesicle (%)	Cell matrix (%)
4°C 30 min						
Au-G	136	10.4	94.1	5.9	0	0
Au-GC	18	1.1	88.9	11.1	0	0
Au-BSA	21	1.5	95.2	4.8	0	0
37°C 3 min						
Au-G	107	13.5	75.7	22.4	1.8	0
Au-GC	15	2.0	86.7	13.3	0	0
Au-BSA	14	0.6	85.7	14.3	0	0
10 min						
Au-G	114	12.5	64.0	18.4	15.8	1.8
Au-GC	21	1.6	85.7	14.2	0	0
Au-BSA	16	1.1	87.5	6.3	6.3	0

The cells were incubated with 1 ml of Au-glucagon (Au-G) or Au-BSA reagent. As control, the cells were incubated with Au-glucagon in the presence of excess (300 μg/ml) unconjugated glucagon (Au-GC).

The present results demonstrate that cell-associated radioactivity (both cell surface and intracellular) increases rapidly during the first 10 min of incubation at 37°C, and then decreases markedly. Similar findings were reported by Sonne et al. (1978) and Barazzone et al. (1980). Although Sonne et al. (1978) considered the decrease in the activity as dissociation of ligand from the receptor, Barazzone et al. (1980) supposed the decrease to be degradation of the ligand by hepatocytes. The present results (assay of glucagon degradation) indicate that the decrease in radioactivity is probably due to degradation of the ligand in the medium by hepatocytes, as suggested by Barazzone et al. (1980). In contrast to the physiological temperature, higher binding of the ligand was maintained at 20°C in this study. This is probably because the ligand is degraded more slowly at 20°C than at 37°C. Dunn et al. (1980) have found that the fusion of endocytotic vesicles and lysosomes does not occur at temperatures below 20°C.

A quantitative autoradiographic study of Barazzone et al. (1980) revealed that, in isolated rat hepatocytes incubated with ^{125}I-glucagon at 37°C for 10 min, most grains were bound to the plasma membrane, and internalization of the ligand occurred by 30 min of incubation. In the present results, labeled-glucagon is internalized already at 3 min of incubation. Mirel et al. (1978) used the Scatchard analysis (Scatchard, 1948) for chemical study of glucagon receptor in hepatocytes, and found that half-maximal binding of ^{125}I-glucagon to the plasma membrane preparations from rat and mouse livers occurred at concentrations of 3.7×10^{-9} M and 4.8×10^{-9} M ^{125}I-glucagon, respectively. This probably corresponds to the difference in the rate of internalization of glucagon between the observations of Barazzone et al. (1980) and ours. Thus, glucagon is internal-

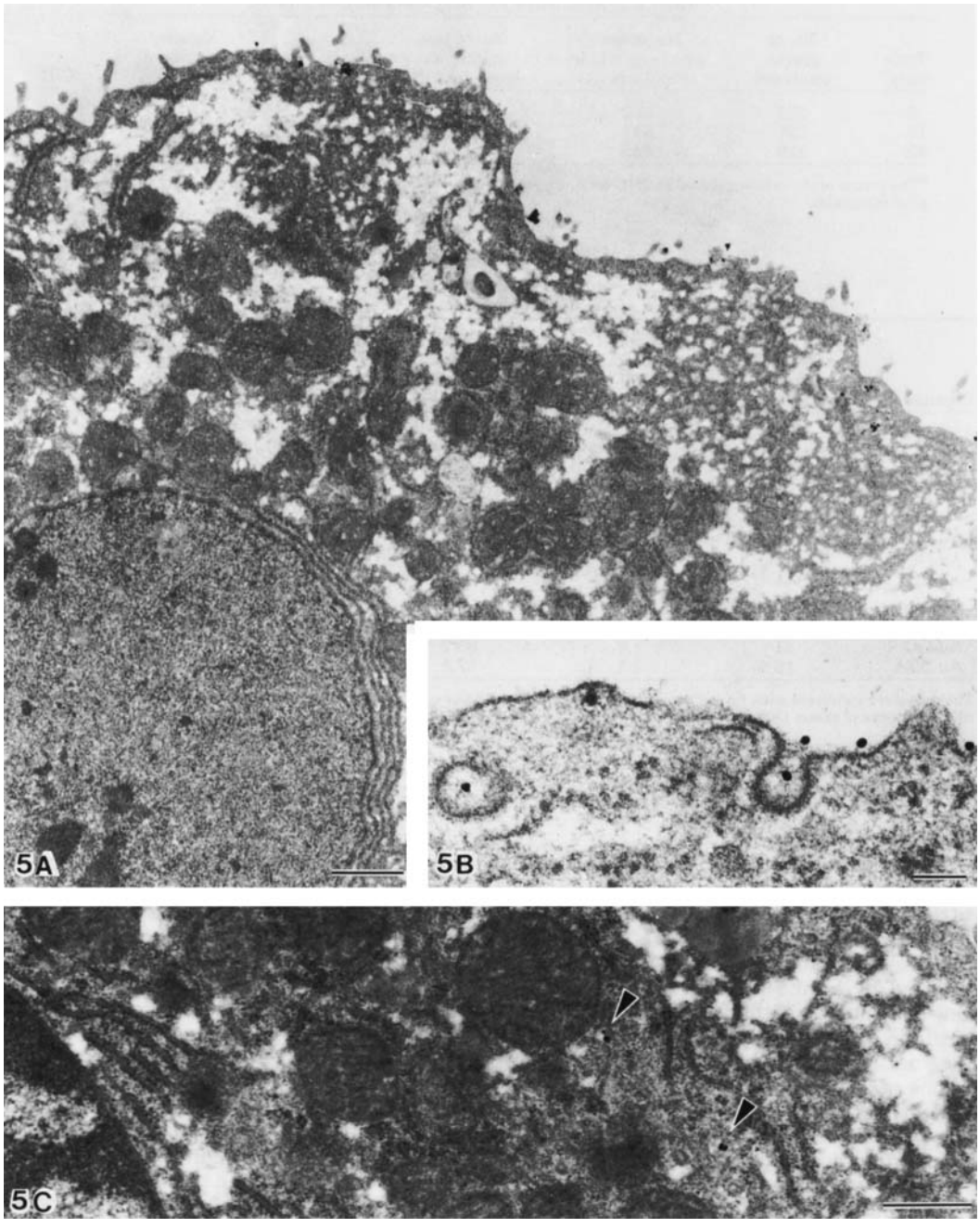


Fig. 5A–C. Portions of hepatocytes incubated with Au-glucagon at 37°C for 5 (A,B) or 10 (C) min. A. Gold particles are seen on cell surface and in cytoplasmic vesicles. ×10,000. Bar = 1 μm. B. High power magnification. Gold particles are localized to the coated pit and in the coated vesicle. ×75,000. Bar = 0.1 μm. C. Gold particles are seen in cytoplasmic vesicles (arrowheads). ×24,000. Bar = 0.5 μm.

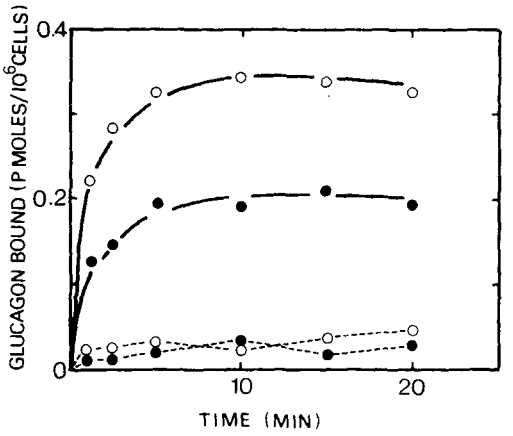


Fig. 6. Time-course binding of 3 nM Au-^{125}I-glucagon (●) or gold-free ^{125}I-glucagon (○) to isolated mouse hepatocytes with (---) or without (—) 300 μg/ml unlabeled glucagon at 37°C. Values are means of two experiments.

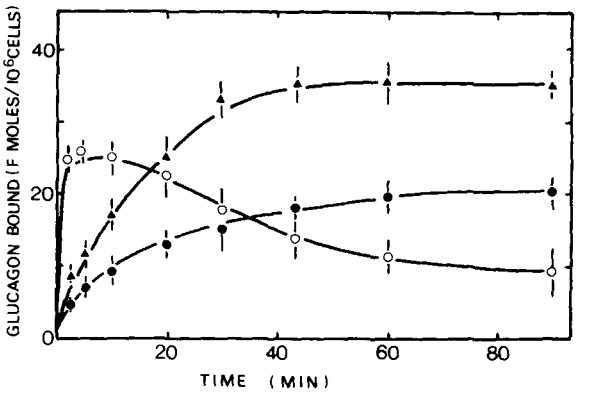


Fig. 7. Time-course binding of 0.4 nM ^{125}I-glucagon to isolated mouse hepatocytes at 4 (●), 20 (▲) and 37°C (○). Nonspecific binding (7–15%) is subtracted. Values are means ± SD for five experiments.

ized more rapidly by mouse hepatocytes than rat hepatocytes. However, the cause of this difference between mouse and rat hepatocytes is unknown.

Au-glucagon contains albumin as a mediator for binding of glucagon and gold particles. Therefore, the presence of the receptor for albumin in hepatocytes is a problem. However, it has been confirmed that albumin uptake into the liver is mainly due to endothelial and Kupffer cells and that only a small amount of albumin is taken up by hepatocytes (Praaning-van Dalen et al., 1981). Further, BSA, added to the incubation medium, serves to inhibit the nonspecific binding of Au-glucagon to hepatocytes.

The uptake of Au-glucagon by the cells in the present biochemical results appears markedly low compared to that of gold-free ^{125}I-glucagon. Glucagon is bound multivalently to one gold particle. Therefore, the amount of label bound to a glucagon receptor is less in Au-glucagon than ^{125}I-glucagon. Horisberger (1981) observed that the amount of gold particles bound to cells was decreased with increasing size of the particles. The author supposed that "large particles could cover many receptors in a cluster," "macromolecules bound to gold particles were less pliable than those in the native state and might not reach receptors," and "some receptors were inaccessible to the larger particles owing to a too narrow spacing of glycoproteins on the cell surface." Further, the internali-

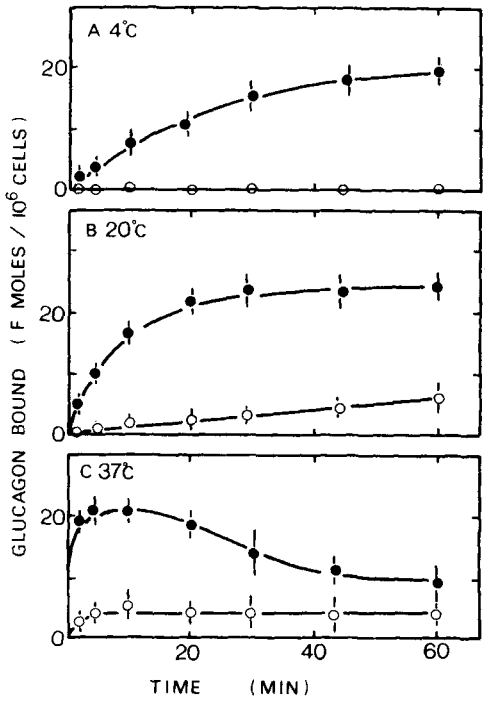


Fig. 8A–C. Time-related change of ^{125}I-glucagon bound to the plasma membrane (trypsin sensitive, ●) or internalized (trypsin resistant, ○) at 4(A), 20(B), and 37°C(C). Nonspecific binding (plasma membrane bound 8–14%, internalized 2–21%) is subtracted. Values are means ± SD for three experiments.

TABLE 3. Analysis of media radioactivity of ^{125}I-glucagon incubated with isolated mouse hepatocytes[1]

Temperature	Percent of ^{125}I-glucagon degraded	
	30 min	60 min
4°C	1.8 ± 0.45	1.7 ± 0.37
20°C	8.9 ± 1.86	16.2 ± 0.92
37°C	42.1 ± 3.88	67.0 ± 5.25
Incubated without cells at 37°C	1.2 ± 0.30	1.3 ± 0.11

[1]An aliquot of the incubation media was analyzed by trichloroacetic acid precipitation. Values are means ± SD for five experiments.

zation of Au-glucagon is somewhat slower than that of ^{125}I-glucagon in the cells incubated at 37°C for 3 or 10 min. This is also probably due to very large size of Au-glucagon compared to that of ^{125}I-labeled glucagon.

Ackerman et al. (1983) observed that tubulo-vesicles containing Au-glucagon were transported to the Golgi region in neutrophils, and the tubulo-vesicles fused directly with lysosomes in monocytes after 10 or 45 min incubation at room temperature. In the present study, however, although internalized Au-glucagon was found in vesicles, such a close relation between the vesicles and Golgi region or lysosomes was not observed. It is not known that the reason for this disagreement is whether our finding is from the cells incubated with Au-glucagon at 37°C for 10 min or the vesicles originally are not transported to Golgi region or lysosomes in hepatocytes. In this study, incubation of the cells with Au-glucagon at 37°C for 20 min or longer was not carried out, because, in our preliminary experiments, a marked dilation of the smooth endoplasmic reticulum, which was indistinguishable from the vesicles, occurred in the periphery of the cells.

Barazzone et al. (1980) reported that internalized ^{125}I-glucagon associated preferentially with lysosomes in rat hepatocytes incubated at 37°C for 30 min. Canivet et al. (1981) found that the lysosomotropic reagents, such as ammonium chloride or chloroquine, partially inhibited the degradation of cell-associated ^{125}I-glucagon. Thus, as suggested by Barazzone et al. (1980), lysosomes were supposed to be involved with degradation of internalized glucagon. Nevertheless, the present results, as well as our previous observations on hepatocytes in vivo (Watanabe et al., 1982; Asada-Kubota et al., 1983), show that the internalized ligand is not exclusively localized to lysosomes.

Similar contradictory results have been reported for insulin. Gorden et al. (1978) and Carpentier et al. (1979a,b) reported that the internalized ^{125}I-insulin was localized and degraded in lysosomes in isolated rat hepatocytes and hepatocytes of intact liver. However, Bergeron et al. (1979) and Goldfine et al. (1978) found that insulin did not preferentially localize to lysosomes in hepatocytes of intact rat liver and cultured human lymphocytes. Further, Renston et al. (1980) showed that the internalized insulin was secreted into the bile. It is possible that glucagon is transported not only to lysosomes but also to other unknown degradation sites by a vesicular transport mechanism. The fates of other ligands than glucagon and insulin in cells appear also various. Ashwell and Morell (1974) and Dunn et al. (1979) reported that asialofetuin was internalized by hepatocytes and transported to lysosomes. Dautry-Varsat et al. (1983) and Harding et al. (1983) found that transferrin was internalized via endocytosis by reticulocytes, and iron was delivered to the cells but iron free transferrin was exocytosed.

LITERATURE CITED

Ackerman, G.A., J. Yang, and K.W. Wolken (1983) Differential surface labeling and internalization of glucagon by peripheral leukocytes. J. Histochem. Cytochem., *31*:433–440.

Asada-Kubota, M., J.Watanabe, S. Kanamura, K. Kanai, and M. Yoshikawa (1983) Binding and internalization of ^{125}I-glucagon in hepatocytes of intact mouse liver. An autoradiographic study. Exp. Pathol., *23*:95–101.

Ashwell, G., and A.G. Morell (1974) The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. Adv. Enzymol. *41*:99–128.

Barazzone, P., P. Gorden, J-L. Carpentier, and L. Orci (1980) Binding, internalization, and lysosomal association of ^{125}I-glucagon in isolated rat hepatocytes. A quantitative electron microscope autoradiographic study. J. Clin. Invest., *66*:1081–1093.

Bergeron, J.J.M., G. Levine, R. Sikstrom, D. O'Shaughnessy, B. Kopriwa, N.J. Nadler, and B.I. Posner (1977) Polypeptide hormone binding sites in vivo: Initial localization of ^{125}I-labeled insulin to hepatocyte plasmalemma as visualized by electron microscope radioautography. Proc. Natl. Acad. Sci. U.S.A., *74*:5051–5055.

Bergeron, J.J.M., R. Sikstrom, A.R. Hand, and B.I. Posner (1979) Binding and uptake of ^{125}I-insulin into rat liver hepatocytes and endothelium. An in vivo radioautographic study. J.Cell Biol, *80*:427–443.

Canivet, B., P. Gorden, J.-L. Carpentier, L. Orci, and P. Freychet (1981) Glucagon degradation in isolated rat hepatocytes: Effect of ammonium chloride and chloroquine. Mol. Cell. Endocrinol., *23*:311–320.

Caro, L.G., R.P. Van Tubberghen, and J.A. Roll (1962) High resolution autoradiography. 1. Methods J. Cell Biol., *15*:173–188.

Carpentier, J.-L., P. Barazaone, P. Freychet, A. Le Cam, and L. Orci (1979a) Intracellular localization of ^{125}I-labeled insulin in hepatocytes from intact rat liver. Proc. Natl. Acad. Sci. U.S.A., *76:*2803–2807.

Carpentier, J.-L, P. Gorden, P.Freychet, A. Le Cam, and L. Orci (1979b) Lysosomal association of internalized ^{125}I-insulin in isolated rat hepatocytes. Direct demonstration by quantitative electron microscopic autoradiography. J. Clin. Invest., *63:*1249–1261.

Dautry-Varsat, A., A. Ciechanover, and H.F. Lodish (1983) pH and the recycling of transferring during receptor-mediated endocytosis. Proc. Natl. Acad. Sci. U.S.A., *80:*2258–2262.

Dickson, R.B., M.C. Willingham, and I. Pastan (1981) α2-Macro globulin adsorbed to colloidal gold: A new probe in the study of receptor-mediated endocytosis. J. Cell Biol., *89:*29–34.

Dunn, W.A., A.L. Hubbard and N.N. Aronson, Jr. (1980) Low temperature selectively inhibits fusion between pinoctyic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. J. Biol. Chem., *255:*5971–5978.

Dunn, W.A., J.H. LaBadie and N.N. Aronson, Jr. (1979) Inhibition of ^{125}I-asialofetuin catabolism by leupeptin in the perfused rat liver and in vivo. J. Biol. Chem., *254:*4191–4196.

Frens, G. (1973) Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nat. Phys. Sci., *241:*20–22.

Geoghegan, W.D., and G.A. Ackerman (1977) Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level: A new method, theory and application. J. Histochem. Cytochem., *25:*1187–1200.

Goldfine, I.D., A.L. Jones, G.T. Hradek, K.Y. Wong, and J.S. Mooney (1978) Entry of insulin into human cultured lymphocytes: Electron microscope autoradiographic analysis. Science, *202:*760–763.

Gorden, P., J.-L., Carpentier, P. Freychet, A. Le Cam, and L. Orci (1978) Intracellular translocation of iodine-^{125}I-labeled insulin: Direct demonstration in isolated hepatocytes. Science *200:*782–785.

Harding, C., J. Heuser, and P. Stahl (1983) Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J. Cell Biol. *97:*239–339.

Horisberger, M. (1981) Colloidal gold: A cytochemical marker for light and fluorescent microscopy and for transmission and scanning electron microscopy. Scanning Electron Microsc., *1981–2:*9–31.

Kahn, R.C., K.L. Baird, J.S. Flier, C. Grunfeld, J.T. Harmon, L.C. Harrison, F.A. Karlsson, M. Kasuga, G.L. King, U.C. Lang, J.M. Podskalny, and E.V. Obberghen (1981) Insulin receptors, receptor antibodies, and the mechanism of insulin action. Recent Prog. Horm. Res., *37:*477–538.

Kaplan, J. (1981) Polypeptide binding membrane receptors: analysis and classification. Science, *212:*14–20.

Klauning, J.E., P.J. Goldblatt, D.E., Hilton, M.M. Lipsky, J. Chacko, and B.F. Trump (1981) Mouse liver cell culture. 1. Hepatocyte isolation. In vitro, *17:*913–925.

Mirel, R.D., H.P. Morris, and R.P. DiAugustine (1978) Membrane receptor function and the loss of glucagon-stimulated adenylate cyclase activity in hepatomas. Endocrinology, *120:*1237–1246.

Pastan, I.H., and M.C. Willingham (1981) Receptor-mediated endocytosis of hormones in cultured cells. Annu. Rev. Physiol., *43:*239–250.

Praaning-van Dalen, D.P., A. Brouwer, and D.L. Knook (1981) Clearance capacity of rat liver Kupffer, endotherial, and parenchymal cells. Gastreoenterology, *81:*1036–1044.

Renston, R.H., D.G. Maloney, A.L. Jones, G.T. Hradek, K.Y. Wong, and I.D. Goldfine (1980) Bile secretory apparatus: Evidence for a vesicular transpot mechanism for proteins in the rat, using horseradish peroxidase and (^{125}I) insulin. Gastroenterology, *78:*1373–1388.

Scatchard, G. (1948) The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci., *51:*660–665.

Schlessinger, J., Y. Schechter, M.C. Willingham, and I. Pastan (1978) Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor of living fibroblastic cells. Proc. Natl. Acad. Sci. U.S.A., *75:*2659–2663.

Sonne, O., T Berg, and T. Christoffersen (1978) Binding of ^{125}I-labeled glucagon and glucagon-stimulated accumulation of adenosine 3′:5′-monophosphate in isolated intact rat hepatocytes. J. Biol. Chem., *253:*3203–3210.

Stäubli, W., W. Schweizer, J. Suter, and E.R. Weibel (1977) The proliferative response of hepatic peroxisomes of neonatal rats to treatment with SU-13 437 (nafenopin). J. Cell Biol., *74:*665–689.

Watanabe, J. M. Asada-Kubota, S. Kanamura, K. Kanai, and M. Yoshikawa (1982) Demonstration of binding and internalization of ^{125}I-glucagon in hepatocytes of intact mouse liver. Okajimas Folia Anat. Jpn., *59:*207–214.

Watanabe, J., S. Kanamura, M. Asada-Kubota, K. Kanai, and M. Oka (1983) Observation of cytochemical alkaline phosphatase acitivity on the plasma membrane of cultured rat hepatocytes by backscattered electron imaging. Anat. Rec., *207:*635–642.

Weibel, E.R. (1979) Stereological Methods 1. Academic Press, London, pp 26–37.

Willingham, M.C., A.V. Rutherford, M.G. Gallo, J,. Wehland, R.B. Dickson, R. Schlegel, I.H. Pastan (1981) Receptor-mediated endocytosis in cultured fibroblasts: Cryptic coated pits and the formation of receptosomes. J. Histochem. Cytochem., *29:*1003–1013.